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ACTIVATION OF PALUDRINE

BY

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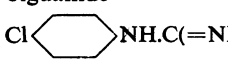
The purpose of this paper is to submit evidence that paludrine has little activity in preventing the development of malaria parasites *in vitro*, but that it develops such activity when administered to the mammalian or avian host or when incubated with minced liver tissue.

Paludrine is N_1-*p*-chlorophenyl - N_5 - *iso*propyl biguanide.



Tonkin (1946), in this laboratory, investigated the effect of adding paludrine to tissue cultures containing exo-erythrocytic forms of *Plasmodium gallinaceum* and found that the parasites developed in the highest concentrations (2–5 mg. per litre) tolerated by the macrophages. Since this concentration is many times greater than that which is believed to be present in the plasma during human therapy (probably about 0.3 mg. per litre), and since Davey (1946) had reported that paludrine was highly effective in curing the exo-erythrocytic infections of *P. gallinaceum* in chickens, as well as the endo-erythrocytic ones, Tonkin's finding was surprising and led to further investigations, which were undertaken with both *P. gallinaceum* and *P. cynomolgi*. A preliminary note describing some of the results was published by Hawking (1947).

Experiments with P. gallinaceum *in tissue culture*

The exo-erythrocytic forms of *P. gallinaceum* were grown in tissue culture at 37° C. by the technique described by Hawking (1945) and used by Tonkin (1946) to study the action of antimalarial compounds. The implants were obtained from the spleen of chickens, at the stage of infection when exo-erythrocytic parasites were present. In the first experiments the cultures were grown in the presence of normal serum for several days, until the development of parasites in them could be demonstrated; the medium to be tested for antimalarial activity was then added and the fate of the parasites was observed by removing cultures at intervals during the next ten days for staining with Giemsa and examination in the usual way. The protocol of a typical experiment is reproduced in Table I. For this experiment serum was

TABLE I

The medium consisted of 20 per cent serum, 20 per cent embryo extract, and 60 per cent Tyrode's solution by volume. The paludrine serum in Nos. 2, 3, and 4 was substituted for all or part of the normal serum. The paludrine in Nos. 6 and 7 was added to the Tyrode's solution. On the 5th day the medium was changed, and fresh medium similar to that originally present was added. Each No. represents two Carrel flasks and most of the observations are based on four cell-colonies (slips), two from each flask. The cultures had grown for 4 days before the experiment began, and the development of parasites in them had been demonstrated.

No.	Contents	Presence of parasites in cultures		
		2nd day	4th day	7th day
1	Control—Normal serum 20%	—	—	Many
2	Paludrine serum 20%	—	A few in one culture. None in another	None—chick not infected
3	Paludrine serum 7%	Some	None	None—chick not infected
4	Paludrine serum 2%	Some	None	None
5	Paludrine 5 mg. per l.	—	—	Toxic to cells
6	Paludrine 2 mg. per l.	—	—	Many
7	Paludrine 1 mg. per l.	—	—	Many

obtained from a fowl to make up the normal fluid culture medium. The fowl was then given 300 mg. paludrine per kg. intramuscularly (a toxic dose), and blood was removed 2¼ hours later to provide the "paludrine serum." (All quantities of paludrine in this work refer to paludrine acetate.)

In this experiment paludrine added directly to tissue cultures had little or no action on the parasites unless the final concentration was so high (e.g., 5 mg. per litre in No. 5) as to be toxic to the cells without which the parasites cannot grow; this reproduces the finding of Tonkin already mentioned. On the other hand the addition of serum from a fowl treated with a large dose of paludrine was highly active, and all the cultures were sterilized, mostly by the 4th day. The survival of parasites for a few days, even in a medium which eventually kills them, is in agreement with the experience of Tonkin, who found that parasites persisted in the presence of sulphathiazole (5–50 mg. per litre) for 4 days but that most were destroyed by the 5th day. The death of the parasites in Nos. 2 and 3 was confirmed by removing fluid from the flasks on the 7th day and injecting it into chicks, which did not become infected. Probably the activity shown by the paludrine-serum in this experiment was particularly high because of the large size of the dose of paludrine given to the fowl. In other experiments the fowl was given 30 mg. per kg. 18 hours and 2 hours before the blood was taken, and the antimalarial action of the serum was not manifested when the concentration of serum in the flask was less than 5 per cent.

These results suggest that paludrine undergoes some modification in the fowl so that it develops an activity against the parasites *in vitro* which was not possessed by the original paludrine. This hypothesis was studied further by incubating paludrine with minced liver tissue and then testing its action on the parasites. The liver was removed aseptically from normal rats and minced finely. About 6 c.c. of minced liver was placed in each of two tubes containing 10 c.c. of Ringer with 0.2 per cent (w/v) glucose; one tube contained 12 mg. paludrine per litre, which when diluted 1 in 6 provided a final concentration of 2 mg. per litre, and the other served as a control. The tubes were incubated at 37° C. for 4 hours and then centrifuged, and the supernatants removed. These provided the "liver extract" and "liver extract plus paludrine," the use of which is illustrated in the typical protocol reproduced in Table II. In this experiment the fluids to be tested for antimalarial activity were added to the Carrel flasks when the cultures were first set up, without allowing a preliminary period for growth (Tonkin, 1946). Serum A was obtained from a normal fowl and it was used as serum for all the cultures unless stated otherwise. Serum B_1 was obtained from a fowl *before* it received paludrine; it was then given two intramuscular injections of 30 mg. per kg. 18 hours and 1 hour respectively before the "paludrine serum," B_2, was withdrawn. In this experiment the paludrine serum showed fairly high activity, since the growth of parasites was prevented (completely or incompletely) by concentrations of 1–4 per cent. Liver extract alone had no activity against the parasites; but paludrine which had been incubated with liver showed fair activity, preventing the growth of all but rare parasites when in a concentration originally of 2 mg. per litre. Actually, it is probable that much of the paludrine had been absorbed by the liver tissue and the real concentration of paludrine (and paludrine derivatives) was much less than this theoretical figure. Other experiments (see Table I) have demonstrated that unchanged

TABLE II

The medium consisted of 17 per cent serum, 4 per cent embryo extract, and 79 per cent Tyrode's solution by volume. The paludrine serum in Nos. 3, 4, and 5 was substituted for all or part of the normal serum (A). The liver extract (with or without paludrine) in Nos. 6, 7, 8, and 9 was substituted for part of the Tyrode's solution. Each No. represents two Carrel flasks and most of the observations are based on four cell-colonies (slips).

No.	Contents	Concentration % (v/v)	Presence of parasites on 5th day
1	Serum A Control	17	Many
2	Serum B_1 Control	17	Very many
3	Paludrine serum B_1	17	None
4	Paludrine serum B_2	4	Few and degenerate
5	Paludrine serum B_2	1	None
6	Liver extract	17	Very many
7	Liver extract + paludrine	17 + 2 mg. per l.	None in 2 cultures, 1 parasite in a 3rd culture, and 2 in a 4th
8	Liver extract + paludrine	4 + 0.5 mg. per l.	Fair number
9	Liver extract + paludrine	1 + 0.12 mg. per l.	Few

paludrine is inactive in a concentration of 2 mg. per litre.

After the tissue culture experiments had been completed, chemical estimations were made on the concentration of paludrine present in the serum of two fowls, each of which had received two intramuscular injections of 30 mg. paludrine per kg. 18 hours and 1 hour respectively before the blood was withdrawn. The concentrations found were 11 mg. and 27 mg. per litre respectively, average 19±11.3 mg. per litre. As the serum constituted only 17 per cent of the fluid present in the flasks of Table II, the highest concentration of paludrine present in the medium containing "paludrine-serum" (Flask 3) would be about 3.2 mg. per litre; while the medium in Flask 5 (which contained only 1 per cent paludrine serum and which prevented growth of the parasites) would contain 0.19 mg. per litre. These figures may be compared with the concentration of 2 mg. unchanged paludrine per litre which is inactive.

Experiments with P. cynomolgi

P. cynomolgi was cultured *in vitro* at 37° C. by a modification of Bass and John's technique. Briefly, about 15 c.c. blood was withdrawn from a rhesus monkey (A) infected with *P. cynomolgi* at a time, preferably before noon, when most of the parasites (95 per cent) were present as small rings. The blood was defibrinated by shaking with glass beads. It was centrifuged, most of the serum was removed, the corpuscles being resuspended in the remainder. Sufficient 10 per cent (w/v) glucose solution was added to the serum to make the concentration of added glucose 0.2 per cent. The cultures were made in small flat-bottomed tubes, 6 cm. high by 0.6 cm. internal diameter, which were closed by rubber bungs. Each tube contained 0.6 c.c. monkey serum, 0.1 c.c. Ringer's solution, and 0.05 c.c. suspension of parasitized red blood corpuscles. The corpuscles formed a thin layer on the floor of the tube. Serum A was obtained from Monkey A or from another normal

TABLE III

Serum A was obtained from an untreated monkey which provided the parasitized blood. Serum B_1 was taken from a second monkey before it received paludrine. Serum B_2 was taken from the second monkey at 11 a.m., when it had received paludrine 50 mg. 18 and 2 hours previously. In tubes 9–11 the dilutions were made with serum A. Tubes 12–19 contained serum A (0.6 c.c.) plus 0.1 c.c. of liver extract (incubated with or without paludrine 14 mg. per litre) pure or in various dilutions made with Ringer. At the beginning of the experiment all the parasites contained only one piece of chromatin, and 95 of them were young rings; there were no gametocytes.

Tube	Contents	Per cent distribution of parasites according to number of pieces of chromatin					
		1	2–3	4–5	6–8	9–16	Degenerate
1	Control, serum A	26	22	12	16	24	0
2	,, ,, ,,	32	18	14	10	26	0
3	Control, serum B_1	28	22	18	10	22	0
4	,, ,, ,,	36	16	4	16	28	0
5	Paludrine, added to serum A, 2 mg. per l.	26	23	15	11	25	0
6	Paludrine, added to serum A, 1 mg. per l.	35	25	10	10	20	0
7	Serum B_2 from monkey treated with paludrine, pure	42	18	6	4	0	30
8	—ditto—pure	54	28	4	0	0	14
9	—ditto—diluted 1 in 4	48	40	8	0	0	4
10	—ditto—diluted 1 in 16	28	48	4	0	0	20
11	—ditto—diluted 1 in 64	69	22	3	0	0	6
12	Liver extract added, pure	23	42	15	7	13	0
13	—ditto—diluted 1 in 4	15	25	9	6	12	33
14	—ditto—diluted 1 in 16	35	20	15	10	20	0
15	Paludrine + liver extract, pure (2 mg. per l.)	4	6	0	0	2	88
16	—ditto—pure (2 mg. per l.)	10	0	0	0	0	90
17	—ditto—diluted 1 in 4 (0.5 mg. per l.)	52	22	6	4	2	14
18	—ditto—diluted 1 in 16 (0.125 mg. per l.)	48	24	4	0	0	24
19	—ditto—diluted 1 in 64 (0.03 mg. per l.)	36	24	16	8	16	0

monkey. A sample of serum (B_1) was collected from another monkey (B). This served as a control to exclude non-specific antimalarial activity. Monkey B was then treated with paludrine, receiving usually two intramuscular injections of 50 mg. for a 3 kg. animal at 5 p.m. and 9.30 a.m.; two hours after the last injection blood was collected from a vein and defibrinated; this provided serum B_2 for the cultures. In the experiments with liver extract paludrine was incubated with minced liver exactly as described above. The concentration of paludrine used, however, was 14 mg. per litre, so that when 0.1 c.c. was added to 0.6 c.c. medium in the culture tube the final theoretical concentration would be 2 mg. per litre. In the tubes to which paludrine was added the paludrine was dissolved in Ringer's solution and added in place of the normal Ringer's solution (0.1 c.c.) in the control tubes. Precautions to maintain sterility were observed throughout. The tubes were incubated at 37° C. After 20 and 42 hours a small sample of the corpuscles was removed by a pipette and smeared out to form thin blood films which were fixed in alcohol and stained with Giemsa. The parasites were examined and classified according to the number of pieces of chromatin which they contained, the identity of the slide being unknown to the investigator during examination.

The protocol of a typical experiment is reproduced in Table III, which shows:

(1) That many of the parasites develop normally (as shown by the division of chromatin) in the presence of serum from the monkey which supplied the parasitized corpuscles (tubes 1 and 2), serum from a second monkey (tubes 3 and 4), or liver extract (tubes 12–14).

(2) That the development of the parasites is not prevented by the addition of paludrine to the cultures sufficient to produce a concentration of 1 or 2 mg. per litre (tubes 5 and 6); in other experiments the parasites developed normally in a concentration of 20 mg. paludrine per litre.

(3) That the development of the parasites is almost completely prevented by serum from a monkey which has received paludrine during the previous 20 hours; this serum is active even when diluted 64 times (tubes 7–11). The parasites in the tubes containing activated paludrine showed various degeneration changes which are described below.

(4) That the development of parasites is similarly prevented by the addition of paludrine which has been incubated with liver (tubes 15 and 18), although the addition of liver extract alone does not inhibit development (tubes 12–14). Inhibition occurs when the paludrine + liver extract is diluted 16 times (tube 18; paludrine theoretically 0.12 mg. per litre) but not when diluted 64 times (tube 19; paludrine theoretically 0.03 mg. per litre).

After these experiments had been completed chemical estimations were made of the concentration of paludrine present in the serum of three monkeys which had been previously treated with paludrine, each receiving 50 mg. per monkey 18 hours and 2 hours before the serum was obtained. The results obtained, using a modification of the method of Spinks and Tottey (1946), are shown in Table IV.

TABLE IV

Monkey	Weight (kg.)	Dose (mg. per kg.)	Blood paludrine (mg. per litre)
1	5.8	8.6	20
2	2.5	20 0	34
3	3.6	13.9	12

The average concentration of paludrine in the serum of a monkey receiving 50 mg. of paludrine as above was thus 22 ± 10.7 mg. per litre. This level is approximately equal to the highest concentration of paludrine (20 mg. per litre) tested in the control tubes during this work; as recorded above, 20 mg. per litre of paludrine solution did not affect the normal development of the parasites. The medium in Tube 11 of Table III was fully active in preventing the development of the parasites although it contained only 1 part of paludrine serum in 64—i.e., probably about 0.3 mg. paludrine per litre.

The conclusions derived from this work are similar to those derived from the experiments with the tissue cultures of *P. gallinaceum*, viz. that paludrine itself (even in a concentration of 20 mg. per litre) is not active in preventing the development of the parasites *in vitro* but that it becomes active when exposed to the cells of the body (as in the treated monkey) or to minced liver tissue.

In a further experiment the supernatant fluid from the incubated tube of paludrine plus liver tissue was heated to 100° C. for 20 minutes in order to precipitate all the protein, which was removed by filtration. This filtrate was as active as the unheated supernatant, which indicates that the active derivative of paludrine is probably not bound to protein, and is probably not thermolabile.

Attempts were made to develop a simple technique for testing the activity of paludrine derivatives *in vitro* by incubating blood containing endo-erythrocytic forms of *P. gallinaceum* with paludrine serum or paludrine + liver extract for several hours and then injecting it into chickens to test for infectivity. The results were very irregular, and most of the specimens were able to infect chickens in spite of exposure to paludrine or its hypothetical products. Accordingly these attempts were abandoned.

Morphological description of degenerate parasites

Examination of preparations of *P. cynomolgi* from cultures in which the parasites had been subjected to the action of activated paludrine showed that, in addition to a failure of development to mature schizonts, the parasites suffered a series of degenerative changes.

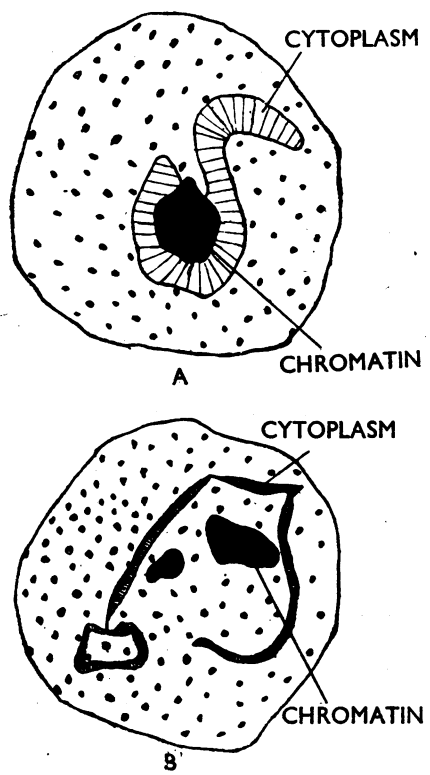


FIG. 1.—Malaria parasites (*P. cynomolgi*) showing degeneration produced by paludrine serum. A. The chromatin is small and dark and the cytoplasm is thinned out and translucent. B. The chromatin is condensed into two dark masses; the cytoplasm is disrupted into narrow strands scattered throughout the corpuscle. Magnification about 8,000.

A few of the parasites were small, containing one piece of very darkly staining chromatin and having a minimum of cytoplasm, also darkly stained, closely packed round the chromatin. Others showed the same small mass of dense chromatin, but had a varying amount of poorly stained pinkish cytoplasm thinned out and almost translucent, so that it was difficult to differentiate from the surrounding corpuscle. An example of this type of parasite is illustrated in Fig. 1A. In this particular specimen the cytoplasm was thin and translucent, but not so distended as in others, in which, indeed, the cytoplasm occupied almost the whole corpuscle. Many parasites, however, exhibited a more characteristic series of degenerative changes than those just described. A typical example is shown in Fig. 1B. The chromatin was condensed into one or more very darkly staining lumps. The cytoplasm was disrupted, and was often arranged in narrow dark-staining strands, which were scattered throughout the corpuscles and woven sometimes into fantastic patterns; frequently the strands were completely separated from the chromatin masses, which were left denuded of any cytoplasmic covering. This series of changes was more common in samples withdrawn from culture tubes after incubation for 42 hours than in samples withdrawn after only 20 hours. The parasitized red blood corpuscles showed the stippling usual with *P. cynomolgi* infections. This series of degenerative changes observed *in vitro* may be compared with the changes undergone by *P. vivax* when subjected to the action of paludrine *in vivo* (Mackerras and Ercole, 1947).

DISCUSSION

The experiments described have shown that paludrine in a concentration of 20 mg. per litre has no apparent action *in vitro* on endo-erythrocytic forms of *P. cynomolgi*; similarly a concentration of 2 mg. per litre has no action on the exo-erythrocytic forms of *P. gallinaceum*. If, however, paludrine has been previously exposed to the action of body cells, either by injecting it into a monkey or fowl and collecting the serum, or by incubating it with minced rat liver, marked antimalarial action can be demonstrated. The simplest explanation for these facts is that paludrine undergoes some chemical modification which converts it into an active compound. This hypothesis would explain the contradictory findings of Tonkin (1946) that paludrine had no antimalarial action when added to tissue cultures of exo-erythrocytic forms of *P. gallinaceum*, and of Black (1946), who reported that serum from a patient treated with

paludrine arrested the development *in vitro* of trophozoites of *P. falciparum*. Marshall (1947) has reported that paludrine solutions are active in inhibiting the uptake of oxygen by *P. gallinaceum in vitro*; but the concentrations of paludrine required to produce this effect were 10^4 to 10^5 times as great as those used in our experiments (1 in 3,000 to 1 in 6,000 as compared with 1 in 1,000,000 to 1 in 10,000,000). No work has yet been reported on the corresponding activity of paludrine after activation by the living animal or by liver extract.

A similar conversion to an active substance occurs with tryparsamide and other pentavalent arsenicals which are converted by cells from the inactive pentavalent to the active trivalent state. With paludrine it is not easy to suggest the nature of the hypothetical chemical modification. According to Acheson, King, and Spensley (1947) it is unlikely that activation depends on the conversion of paludrine into a benziminazole. Our present information indicates that this activation can be carried out by the whole organism or by minced liver; it cannot be produced by the red blood corpuscles or by the macrophages and fibroblasts present in tissue cultures from the spleen. The further study of the activation of paludrine requires a simpler and more convenient technique for measuring the antimalarial action of compounds *in vitro*. The methods used in the present work, although adequate to demonstrate the occurrence of activation, are rather laborious and insensitive for use in a detailed investigation of the phenomenon.

The conversion of paludrine by cells into an active derivative is of importance in the study of the action of paludrine *in vitro*, the relation of chemical structure to activity, the search for more active antimalarial compounds, the interpretation of chemical estimations of the blood concentrations of paludrine, and other similar questions; most of this is obvious and does not require elaboration.

SUMMARY

1. No demonstrable antimalarial action is exerted *in vitro* on cultures of the exo-erythrocytic forms of *Plasmodium gallinaceum* by paludrine in concentrations of 2 mg. per litre, or on endo-erythrocytic forms of *P. cynomolgi* by paludrine in concentrations up to 20 mg. per litre; these concentrations are higher than those which are commonly reached in the blood during human therapy.

2. Serum from a fowl or monkey, which has recently received paludrine, exercises a pronounced action on these cultures, preventing the development of the parasites; cultures of *P. gallinaceum* are destroyed.

3. Paludrine which has been incubated with minced liver has a similar pronounced antiplasmodial action *in vitro*.

4. These phenomena suggest that paludrine itself is not active against plasmodia but that it can somehow be modified by the body or by liver cells, so that it becomes actively plasmodicidal.

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